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EARLY PHASE INTERACTIONS OF TOLUENE WITH MEMBRANES: A
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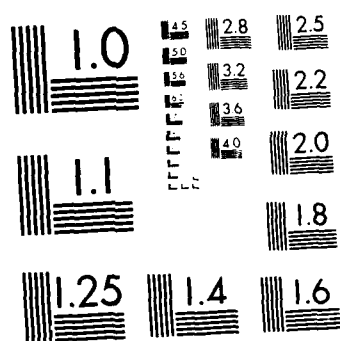
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Madelene Weinbarger
Contracting Officer
Attn: PKD (Anna Beachem)
Department of the Air Force
Air Force Office of Scientific Research
Bolling Air Force Base, DC 20332

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EARLY PHASE INTERACTIONS OF TOLUENE WITH MEMBRANES:

A STRUCTURAL AND FUNCTIONAL EVALUATION

Technical Report

Prepared by

D. James Morre

Department of Medicinal Chemistry and Pharmacognosy
Purdue University, West Lafayette, IN 47907

Submitted through

Purdue Research Foundation
Attn: Ms. Chandrea Lightfoot
Office of Contracts & Grant Business Affairs
Hovde Hall of Administration
Purdue University
West Lafayette, IN 47907

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D. James Morre
D. James Morre, Professor
Principal Investigator

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Objectives

The principal objective of the research conducted was to define the sub-cellular site(s) or target(s) of action of the aromatic hydrocarbon toluene. Confirmed target sites were then to be investigated in detail to elucidate possible mechanisms of toluene action in perturbing membrane structure that might be related to either an enhancement or loss of membrane function.

Approach

The approach to the conduct of this investigation was organized under three problems. Work has proceeded sequentially beginning with target identification under Problem I to target verification under Problem II. The work is now focusing on basic mechanistic studies under Problem III.

1) Morphological observations with cells in culture.--In experiments using KB and L-cells in culture, viability was maintained at 25 ppm toluene but growth was slowed. L-cells were selected to begin a survey of changes in electron microscope morphology (Figs. 1 and 2). Several hundred cells were examined. The most obvious and striking changes involve the plasma membrane at the cell surface (Fig. 1A). Untreated cells had many surface protuberances (microvilli). After 15 min of exposure to 25 ppm toluene, the surface became smooth (Fig. 1B). There was some tendency for the cells to round up and to show changes in the orientation of the Golgi apparatus by 30 min (Fig. 1C). With a single exposure, the effects are reversible and by 2 h (Fig. 2D), a nearly normal surface morphology was restored including the numerous microvilli.

With cells exposed to 100 ppm toluene, the morphological responses after 5 min were similar to those observed after 30 min with 25 ppm toluene with regard to surface morphology (Fig. 2). This concentration, which eventually was toxic, resulted in many additional ultrastructural modifications. These included distention of the nuclear envelope, dilation of the luminal space of the endoplasmic reticulum, disorganization of the Golgi apparatus and swelling of mitochondria.

Under this problem, several test systems developed in our laboratory for toxicological evaluation of target sites of membrane active substances were employed. The basic approach was to subject each tissue to a graded series of toluene concentrations for varying periods of time after which the material was prepared for electron microscopy under conditions developed to yield accurate and reproducible evaluations. Comparisons were to identical tissues treated in a similar fashion in the absence of toluene. Gross and subtle morphological changes were noted indicative of an activity target using the following test systems:

- Test System 1. Primary Rat Hepatocytes in Culture and Liver Slices In Vitro
- Test System 2. Cultured BHK, KB, L, RLT-28 and L Cells
- Test System 3. Outer Cap Cells of the Maize Root Tip
- Test System 4. Guinea Pig Testes Tubules
- Test System 5. Frog Epidermis (recently added).

Most of the work has been with test systems 1 and 2. Using these systems, we find cell mortality with extended exposure to between 500 and 1000 ppm toluene with 25 ppm toluene being about the lowest concentration at which toxicity can be detected. At 25 ppm with most cells, growth is slowed but viability is maintained.

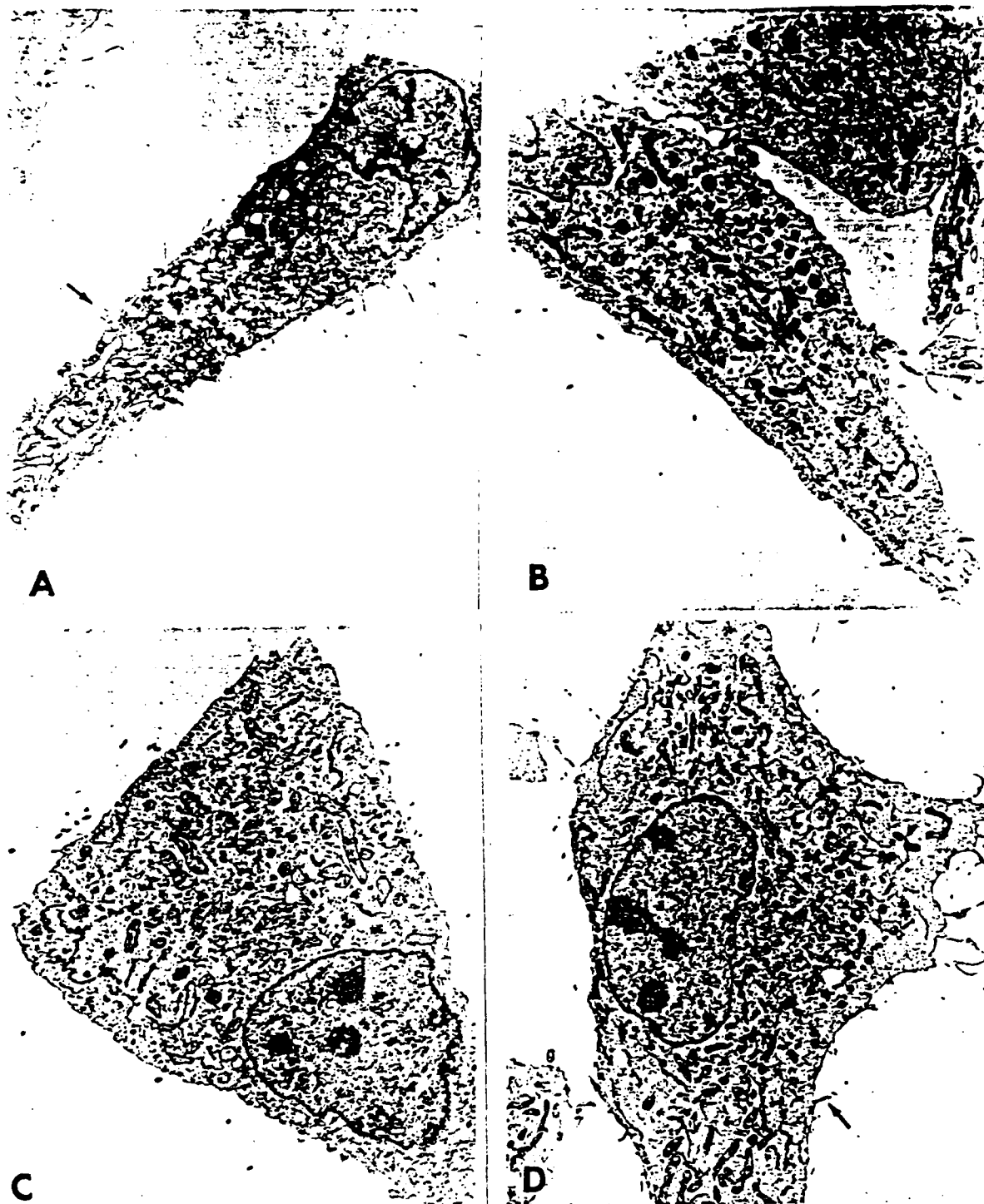


Fig. 1. Electron micrographs of L cells illustrating cell surface (plasma membrane changes observed following a single exposure to 25 ppm toluene contained in the cell culture medium. A. No toluene (control). Note numerous surface protuberances. B. 25 ppm toluene for 15 min. The cell contour is now smooth. C. 25 ppm toluene for 30 min. The cell contours remain smooth and the cells begin to round with alterations in the form and position of the Golgi apparatus. D. 25 ppm toluene for 2 h. As the toluene is lost from the medium, the effects disappear, the normal morphology returns including the typical form and placement of the Golgi apparatus and the numerous surface protuberances. Figs. A, B and C X 6,000. Fig. D X 4,500.

However, the most obvious, early, low-dose responses were those described for the plasma membrane of the cell surface (Fig. 2).

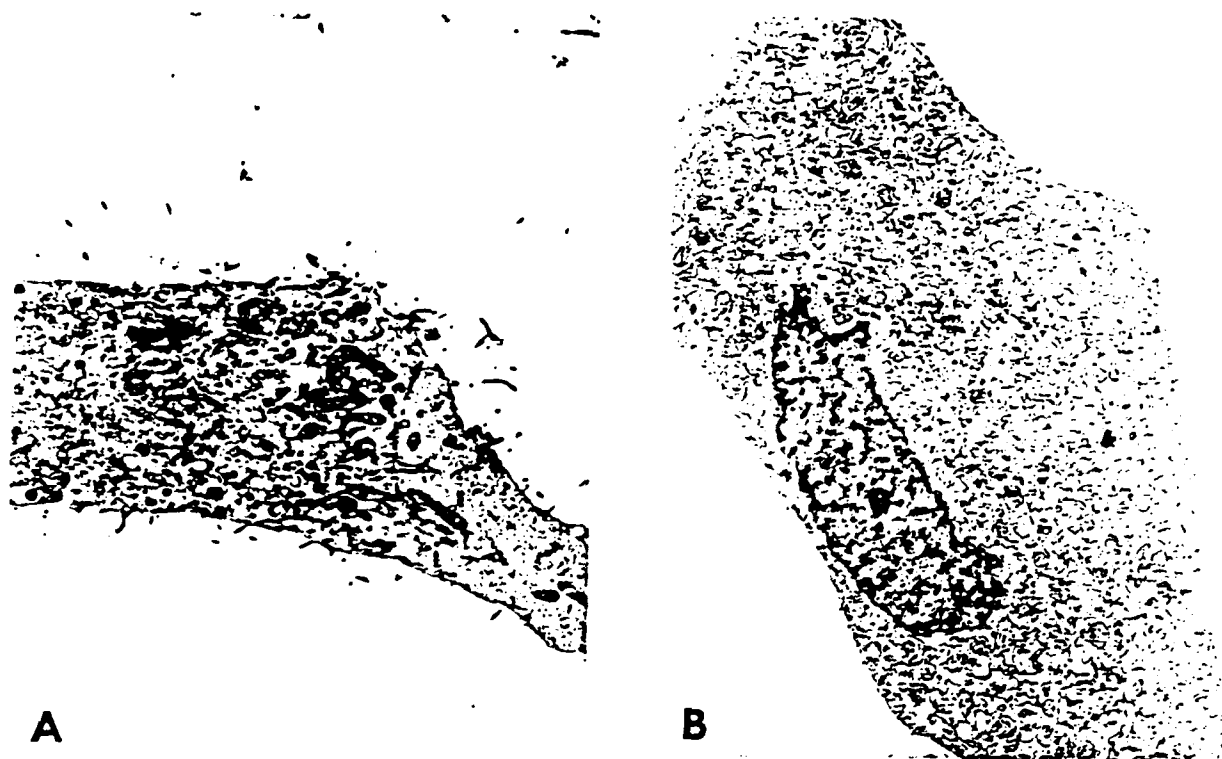


Fig. 2. Electron micrographs of L cells illustrating cell surface-plasma membrane changes observed following a single 5 min exposure to 100 ppm toluene contained in the culture medium. The treated cells responded rapidly and presented a surface morphology after 5 min comparable to that observed after 30 min with 25 ppm toluene. This dose of toluene was toxic and results in many additional ultra-structural modifications involving membranes (see text). Fig. A x 4,500. Fig. B x 6,500.

2) Effects of the sodium ouabain-inhibited Na^+, K^+ ATPase.--Experiments have been conducted with the ouabain-inhibited Na^+, K^+ ATPase of liver plasma membranes and cultured cells to show a marked response of this activity in terms of its sensitivity to ouabain. We have used the activity of a K^+ -p-nitrophenylphosphatase (K^+ -pNPPase) as a measure of the K^+ -stimulated, ouabain inhibited, Na^+ pump ATPase both in situ and with cell fractions. These two activities correlate very closely in other systems. The greater sensitivity of the nitrophenyl substrate make possible activity measurements with smaller amounts of plasma membranes.

Data for rat liver are shown in the Figure although similar results have been obtained for cultured cells and membranes from cultured cells as well as from plasma membranes isolated from amphibian epidermis. What we find is that in the presence of very low doses of toluene (maximal response at about 25 ppm), there is a loss of ouabain inhibition. This loss is greatest in the absence of potassium and least in the presence of potassium (Fig. 3). Basal activity ATPase activity of the membrane preparations is relatively unaffected by toluene as is basal pNPPase activity. The effect is specifically on the ouabain-inhibited component. In fact, in the absence of potassium, ouabain stimulations of the activity are recorded when toluene is present. These findings suggest a specific effect of toluene on the Na^+

pump ATPase of the cell surface perhaps involving a change in the conformation of the activity at or near the ouabain site involved in potassium transport.

3) Metabolic labeling of tissue slices.--In metabolic labeling experiments, we have utilized slices from livers of 200 g male Wistar rats. A dosing schedule was developed using a sealed chamber. At the end of the toluene treatment (15, 30 or 60 min),

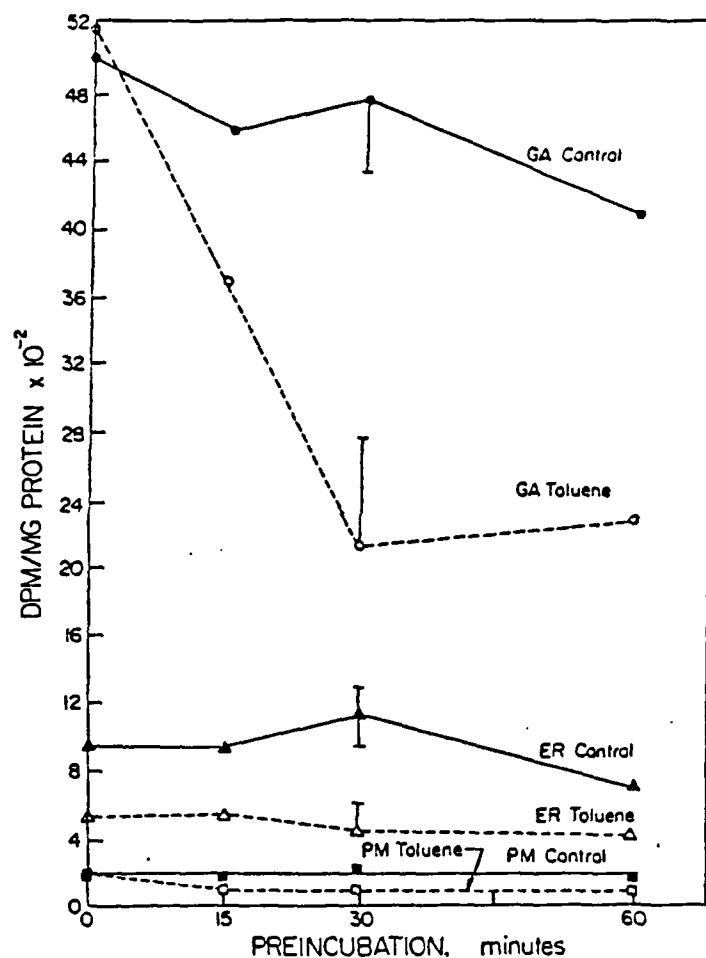


Fig. 4. Time of preincubation with 500 ppm toluene on a subsequent 20 min incubation with $[^3\text{H}]$ -leucine of rat liver slices *in vitro* at 37° . A maximum effect with Golgi apparatus (GA) and plasma membrane (PM) was obtained with a 30 min preincubation whereas with endoplasmic reticulum (ER), an effect was observed even without preincubation.

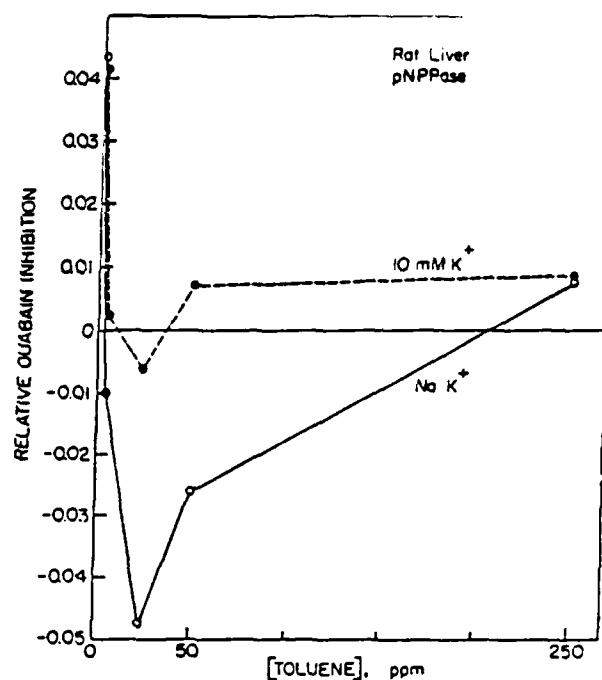


Figure 3. Loss of ouabain inhibition of K^+ -stimulated p-nitrophenylphosphatase (pNPPase) of rat liver plasma membranes as a function of toluene concentration.

radioactive precursors (e.g. $[^3\text{H}]$ -leucine) were administered for an additional 20 min to measure metabolic activity. Then each of the various membrane fractions was isolated and analyzed for incorporation of radioactivity (nuclei, Golgi apparatus, endoplasmic reticulum, lysosomes, mitochondria, and plasma membranes) (Fig. 4 and Appendix Table I).

Incorporation of radioactivity from $[^3\text{H}]$ -leucine into the various membrane fractions was reduced considerably in Golgi apparatus, endoplasmic reticulum and plasma membrane by pretreatment with 500 ppm toluene (Fig. 3). A maximum effect on Golgi apparatus and plasma membrane was obtained with a pretreatment time of 30 min whereas with the endoplasmic reticulum, inhibition was recorded just with toluene pretreatment during the 20 min time of isotope incorporation (Fig. 4).

The dose dependency shown in Fig. 5 and Table I indicated a near maximum response at 500 ppm for a 30-min pre-incubation. Values for 0 ppm (Control) are the same as for Table I.

As seen throughout these data (Appendix Table I and Table I) at 500 ppm toluene, endoplasmic reticulum exhibited a marked and rapid reduction in the amount of [3 H]-leucine incorporated. Subsequently, reductions were seen as well in Golgi apparatus and plasma membrane. Incorporation into nuclei was much less affected while incorporation into mitochondria was affected similarly to that of endoplasmic reticulum. Labeling of lysosomes was investigated in a separate series of experiments and was found not to be affected by toluene.

In subsequent studies, an incubation time of 30 min (Fig. 5; Appendix Table I; and Table I) and a toluene concentration of 500 ppm (Fig. 5; Table II) were judged to provide a maximum response with slices of rat liver.

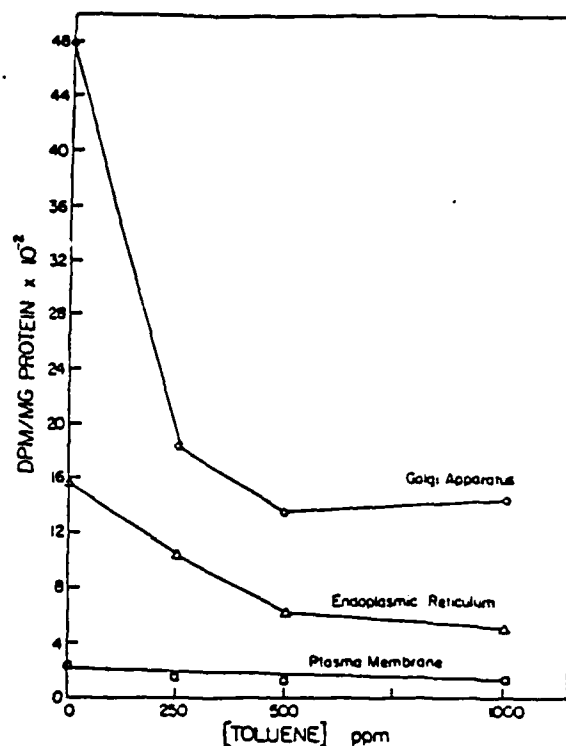


Fig. 5. Effect of toluene concentration during a 30 min preincubation on a subsequent 20 min incubation with [3 H]-leucine of rat liver slices at 37°. Maximum effect with a 30 min incubation was at 500 ppm

Table I

Effect of toluene concentration during a 30 min preincubation on subsequent incorporation during 20 min of [3 H]-leucine into rat liver slices in vitro at 37°. Results are single determinations except for 0 ppm (= control) which are the average of 3 different preparations \pm standard deviations.

Fraction	Dpm/mg protein			
	0 ppm	250 ppm	500 ppm	1000 ppm
Total Homogenate	13,926 \pm 590	15,550	12,632	14,162
Golgi Pellet	1,477 \pm 272	385	375	646
Golgi Apparatus	9,604 \pm 2,462	3,671	2,714	3,020
Supernatant	44,256 \pm 7,961	47,240	45,500	33,846
Plasma Membrane	261	182	146	146
Mitochondria	471 \pm 145	198	133	292
Nuclei	673 \pm 195	352	1,350	675
Nuclei-II	386 \pm 205	444	147	750
ER ₀	1,201 \pm 198	1,074	2,667	1,100
ER ₁	1,483 \pm 1,076	1,222	483	224
ER ₂	1,810 \pm 1,672	(612)	1,287	666

Whereas, overall incorporation during the 7.5 min pulse was less, there was no difference in the rate of passage through the Golgi apparatus or in the time or rate of appearance in the plasma membrane (Fig. 6).

To study the effects of toluene on liver lysosomes, lysosomes were purified by centrifugation in Percoll gradients. When preincubated for 30 min with 500 ppm toluene latency was decreased, lability was increased. In contrast, 500 ppm toluene added directly to preparations of purified lysosomes or to crude preparations containing lysosomes had little or no effect, thus pointing again to a general response of the exocytic export route via Golgi apparatus to toluene.

In long-term studies with cultured cells at lethal doses, distinct nuclear changes precede or accompanied cell death. These changes included a distinct pattern of chromatin clumping in response to the toluene treatment. This pattern was restricted to toluene levels that were lethal and was not observed at sublethal toluene doses. In other respects, the cell nucleus was little affected by toluene.

4) A specific response of endoplasmic reticulum-derived transition elements to toluene.--The basis for the inhibition of membrane flux by toluene in liver slices has been the subject of intense investigation during the past six months. Substantial progress has been made both in the identification of the structural basis for the inhibition and in the formulation of a cell-free system for a detailed molecular study of the basis of toluene action on a specific vesicle formation-fusion process critical to cell growth and development.

a. The structural basis for the response.--An understanding of the structural basis for the toluene response requires an appreciation of the complex series of events thought to be involved in the formation of new Golgi apparatus cisternae by the fusion of small 600 nm transition vesicles derived from part-rough (with ribosomes) and part-smooth (lacking ribosomes) elements of the endoplasmic reticulum known collectively as transition elements. This process is illustrated schematically in Figure 7. Blebs form from the endoplasmic reticulum in an energy-requiring process that is aided presumably by the acquisition of a coat protein (not clathrin but perhaps similar to the clathrin coat protein of coated pits and other transport vesicles of endocytosis). The vesicles migrate across the short distance between the endoplasmic reticulum elements and the Golgi apparatus and, at the Golgi apparatus, the small vesicles coalesce to form new Golgi apparatus cisternae. In so doing the vesicles serve as transport vehicles to newly synthesized membranes and perhaps secretory materials as well as from the one compartment to the next in the

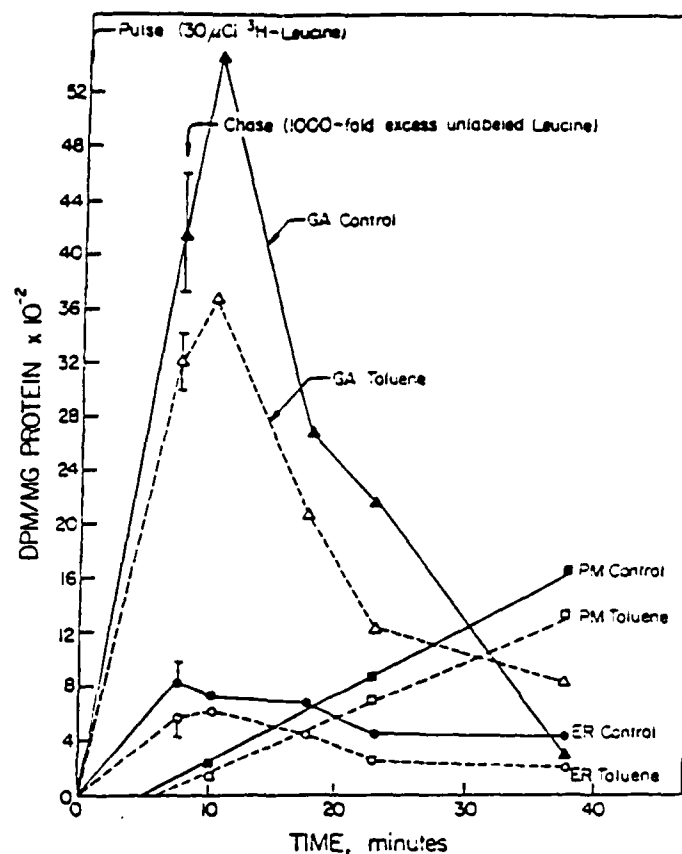


Fig. 6. Flow kinetics of membrane and secretory proteins labeled with a 7.5 min pulse of [³H]-leucine and a subsequent chase with 1000-fold excess of non-radioactive leucine following a 30 min preincubation with 500 ppm toluene. GA - Golgi apparatus. ER - endoplasmic reticulum. PM - plasma membrane. Solid curves are control values. Dotted curves are for liver slices preincubated for 30 min with 500 ppm toluene.

secretory pathway.

In our experiments with liver slices treated with 500 ppm toluene for varying periods of time, we observed subtle changes in Golgi apparatus/transition element organization, consistent with a specific toluene-induced blockage of the formation of the small endoplasmic-reticulum derived vesicles. These changes were observed subsequently in a variety of test systems including cultured cells where the response was seen at toluene concentrations as low as 100 ppm (Figs. 8 and 9). The overall response illustrated in the figures was a general absence of transition vesicles accompanied by a proliferation (filling in or enlargement) of the existing transition elements associated with the Golgi apparatus as expected if transition element formation were blocked. These morphological observations would explain the findings from the metabolic labeling experiments with liver slices where radioactivity appeared to accumulate in the transition vesicle fraction (ER_0) with an accompanying inhibition of transfer of material to the Golgi apparatus and subsequently to the plasma membrane as well.

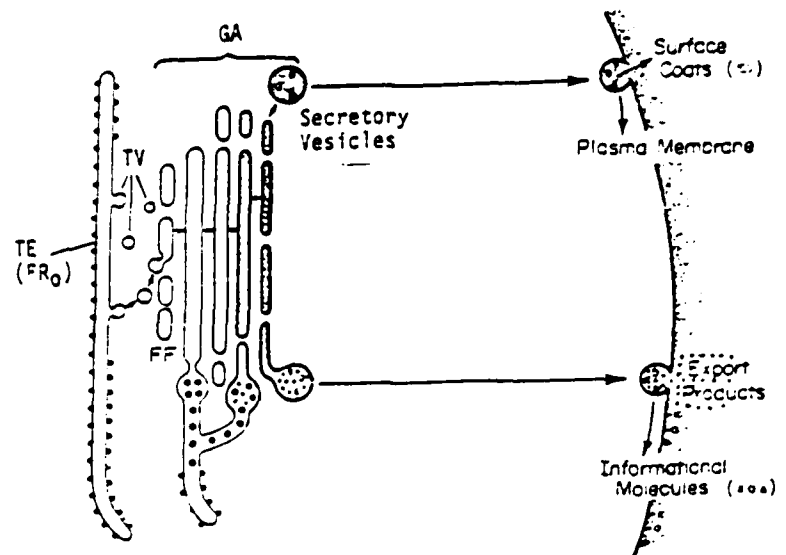


Fig. 7. Diagram illustrating the proposed mechanism for formation of new Golgi apparatus (GA) cisternae by coalescence of transition vesicles (TV) derived from part-rough, part-smooth transition elements TE = ER_0 of the endoplasmic reticulum.



Fig. 8. Electron micrograph of the Golgi apparatus region of a control CHO cell. Note the stacked cisternae of the Golgi apparatus (GA), numerous transition vesicles (small arrows) and the normal appearance of the endoplasmic reticulum elements aligned at the cis Golgi apparatus face (large arrows). Scale bar = 0.2 μ m.



Fig. 9. As in Figure 8 but after a brief exposure to 100 ppm toluene. The appearance of the Golgi apparatus is still relatively normal but transition vesicles are less abundant and the appearance of the Golgi apparatus-associated endoplasmic reticulum is altered in that this membrane system has become much more enlarged and become more elaborate (arrows). Scale bar = 0.2 μ m.

b. Development of a cell-free system.--As detailed in a recent publication, (D.J. Morre, M. Paulik and U. Nowack. 1986. Transition vesicle formation in vitro. *Protoplasma* 132:110-113), we were fortunate to isolate from liver and from liver slices a cell fraction enriched in the vesicle forming-transition elements. These fractions when incubated with ATP and a cytosol-derived protein fraction at 37° responded by the production of putative transition vesicles in vitro. In a subsequent series of investigation, we immobilized Golgi apparatus membranes to nitrocellulose strips and demonstrated transfer of material from the transition elements to the Golgi apparatus membranes presumably via the production of transition vesicles. The process was time and temperature dependent and nearly completely blocked by low concentrations (100 ppm) of toluene.

In subsequent and on-going studies, we have concentrated transition vesicles formed in vitro by the technique of preparative free-flow electrophoresis (Figs. 10 and 11). In this technique where components to be separated are injected as a fine jet into a separation buffer flowing perpendicular to the field lines of an electric field and membranes having different surface charge densities are readily separated. As illustrated in Figure 10, for a primed transition element system

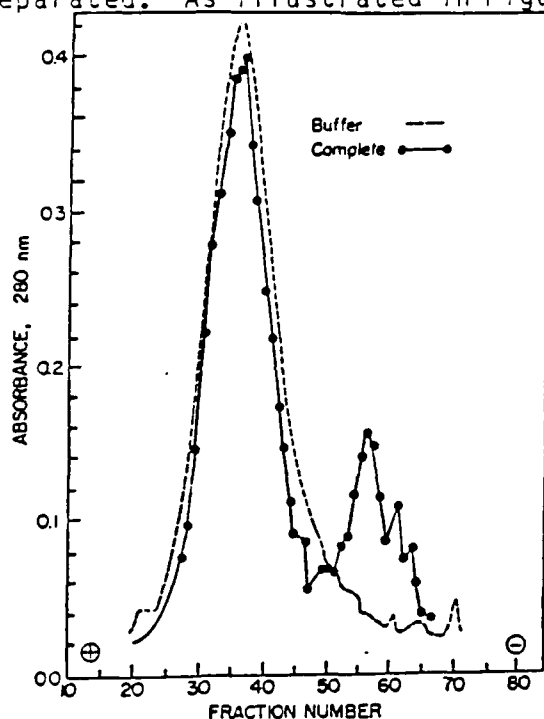


Fig. 10. Free-flow electrophoretic separation of primed (●—● = complete) and unprimed (---- = buffer only) transition elements of rat liver. The small peak centered at about fraction 56 contained the transition vesicles (see Fig. 11). The major peak centered at fraction 36 contained the bulk of the unaltered starting material.

(incubated with ATP, an ATP regenerating system and cytosol), free-flow electrophoresis separates the bulk of the endoplasmic reticulum membranes from a



Fig. 11. Electron micrograph of a transition vesicle preparation by preparative free-flow electrophoresis (e.g. right hand peak of Fig. 10). The many 60 nm vesicles with electron dense, nap-like coatings present are indicated by arrows. These preparations containing 30% or more of the membranes present as transition vesicles, were capable of fusing quantitatively with Golgi apparatus preparations immobilized to cellulose nitrate strips. Scale bar = 0.5 μ M.

second peak nearer the point of sample injection (less electronegative). This peak consisted of numerous small vesicles, 30% or more with morphologies similar to those of the transition vesicles formed in situ (Fig. 11). They were about 60 nm in diameter with electron dense and nap-like surfaces. To equate these vesicles functionally to transition vesicles, the free-flow electrophoresis fractions were made radioactive by metabolic labeling of the tissue slices from which they were derived and were added to non-radioactive rat liver Golgi apparatus immobilized to cellulose nitrate strips. Radioactivity was transferred rapidly and quantitatively with a $T_{1/2}$ of less than 5 min. In contrast to vesicle formation, the coalescence of the already-formed vesicles with Golgi apparatus did not require ATP but was enhanced by cytosol and was unaffected by toluene. Thus toluene effects vesicle formation, not fusion, in this system.

The complete incubation mixture was necessary to obtain the formation of transition vesicle fraction illustrated in Figures 10 and 11. The second peak enriched in transition vesicles was present only in preparations incubated with membranes in the complete system, i.e. ATP and ATP regenerating system together with membranes present, only when incubated with ATP plus ATP regenerating system and in cytosol. However, when toluene also was included during the incubation at final concentrations between 100 and 500 ppm, the formation of these vesicles was completely inhibited. These observations corroborate the results observed in vivo and provide the basis for a convenient test system to probe further the molecular details of toluene action in preventing the flux of membranes between endoplasmic reticulum and the cell surface.

5) Summary.--The most sensitive cell component to toluene was the plasma membrane where a morphological response at and a response in terms of enzymatic activity was observed at 25 ppm both with treatment times of 5 min or less. Thus the plasma membrane is indicated as one important target for toluene intoxication.

A second target identified was that of the transition region between endoplasmic reticulum and Golgi apparatus where transfer of material appears to be rapidly blocked by 100 ppm toluene or lower both in situ and in a cell free system newly developed to study this phenomenon. Subtle changes in membrane cytoskeleton interactions or in membrane fluidity involving boundary lipids of membrane proteins may provide the common denominator between these two points of toluene action at the subcellular level.

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